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Conjugated linoleic acid (CLA) is a naturally occurring component of the food supply that inhibits mammary carcinogenesis. Data obtained on this project indicate: 1) that the anticancer activity of CLA is unlikely to be explained based soley on its antioxidant activity; 2) that CLA is unlikely to inhibit carcinogenesis by directly antagonizing linoleic acid metabolism; 3) that CLA inhibits carcinogenesis irrespective of the presence or absence of the codon 61 mutation in the Ha-ras gene. Candidate genes that are over-expressed specifically in mammary carcinomas have been identified. The effects of CLA on the expression of these genes will be investigated to determined if they are involved in the inhibition of mammary carcinogenesis by CLA.

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Breast Cancer, conjugated linoleic acid, antioxidant activity, lipid peroxidation, oxidative DNA damage, Ha-ras

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Henry J Thompson 10/8/98

PI - Signature / Date

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INTRODUCTION

Programatic Note: A one year no cost extension of this project was requested and approved. The purpose of the extension was to permit completion of data evaluation and the preparation of manuscripts. This is an interim report and does not constitute a final report of findings.

PROPOSAL RELEVANCE. In 1981 Doll and Peto (1) estimated that nutrients and other dietary factors could account for a significant percentage of the risk for epithelial cancers in the United States and recently Doll (2) has suggested that approximately 35% of these cancers may be preventable via changes in dietary behaviors. Of the nutritional and dietary factors considered with regard to the risk for breast cancer, the role that the amount and type of dietary fat and calories play in the disease process has received prominent attention. This work has recently been reviewed (3,4). Two facts that have surfaced in this area of investigation are particularly relevant to the experiments being conducted. First, the level of caloric intake has a prominent effect on mammary tumorigenesis (3) and second, dietary fat has a specific effect on mammary tumorigenesis, but this effect is observed only when caloric intake is ad libitum (3). Our laboratory was one of the first to report the requirement for ad libitum intake for a fat specific effect on mammary tumorigenesis to be manifest (5), an observation that has recently been confirmed by others (6). It appears that this observation applies over a range of dietary fat concentrations. Given that a major health concern in the United States continues to be the consequences of intake of calories in excess of energy needs, it is probable that fat specific effects are being exerted in the U.S. population and other societies in which there is a surfeit of dietary calories.

As part of an overall public health initiative, Americans are being encouraged to eat less and exercise more in order to maintain "ideal" body weight, and to reduce the percent of dietary calories that they consume as fat (7). This advice is given with greatest specificity for prophylaxis of diseases of the heart, but these recommendations also apply to cancer, especially of the breast and colon. In general, it is recommended that dietary fat intake be reduced to $\leq$ 30% dietary calories with $\leq$ 10% provided as saturated fat, 10% as monounsaturated fat and 10% as polyunsaturated fat. An opportunity exists, therefore, to make recommendations about the specific fats that provide these calories. With regard to cancer, a principal interest lies in altering the type of polyunsaturated fatty acids (PUFA) that are being ingested. The question now receiving particular attention is whether all families of PUFA have similar effects on tumorigenesis and if individual fatty acids have selective effects on the mammary gland. ***The program of research being conducted on this grant specifically addresses this issue. We are investigating the cancer preventive activity of a specific fatty acid, conjugated linoleic acid (CLA), and we are studying various mechanisms that may account for its protective activity.***

CLA , a collective term that refers to conjugated dienoic derivatives of linoleic acid, is a naturally occurring substance in dairy products and in animal tissues. In a number of recent publications evidence has emerged indicating that CLA fed in the diet is a potent inhibitor of chemically-induced mammary carcinogenesis in the rat (8-11). This effect of CLA is in sharp contrast to that of linoleic acid which has been shown to stimulate the carcinogenic process in the same tumor model system in a dose dependent manner. Of added interest is the apparent potency of CLA in cancer prevention in comparison to other fatty acids reported to have cancer inhibitory activity. The most prominent among these are the fatty acids in fish oil. However, the amount of fish oil needed for cancer inhibitory activity usually exceeds 10% (w/w) in the diet. Recent work indicates that a level of CLA as low as 0.1% (w/w) was sufficient to produce a significant inhibition of mammary carcinogenesis. Thus, CLA is considerably more potent than any other fatty acid in inhibiting tumor development.

The potential relevance of these observations for cancer prevention in humans is considerable. In a direct extrapolation of the laboratory animal data to a 55 kg person, the amount of CLA required for cancer prevention would be equivalent to 2.8 g per day. The current estimate of CLA consumption per day in the United States is 1 gram. The difference in these values is relatively small. Given that dietary levels of at least 1.5% CLA (w/w) can be fed chronically without adverse consequences, it appears that achieving a protective level of CLA consumption is quite feasible. CLA offers great potential as a preventive agent and could even be provided at effective levels via the food supply either via designer foods or as a dietary supplement.

In the work currently being conducted on this grant we are investigating the biological activity(s) of CLA that accounts for its cancer preventive activity. Our working hypothesis is that CLA affects the processes of clonal expansion and/or clonal selection via modulating genetic and/or epigenetic mechanisms obligatory for, or permissive to the carcinogenic process. This hypothesis is being evaluated by determining the effect of CLA on the expression of molecular markers relevant to the process of mammary carcinogenesis. These investigations may identify critical molecular events that can be targeted for cancer prevention.

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TECHNICAL OBJECTIVES OF THIS PROJECT .

Objective 1. ***Does CLA inhibit the formation of oxidative damage to DNA?***

CLA has been reported to be a potent antioxidant in test tube assays, but its biological activity as an antioxidant is unclear. We have reported that feeding CLA reduced lipid peroxidation in mammary gland measured as malondialdehyde but had no effect on DNA oxidation measured as 8-hydroxydeoxyguanosine (8-OHdG). During the past year we have begun to further examine the effects of CLA on DNA oxidation using methods that enhance sensitivity to detect effects on DNA oxidation. We have also assessed the feasibility of determining if CLA affects the transcriptional activity of the redox sensitive protein, NF-kappa B, the activation of which has been implicated in carcinogenesis.

Objective 2. ***Does CLA alter the process of clonal expansion that occurs in the mammary gland in response to carcinogenic insult?***

During this reporting period a new approach to assessing the effects of CLA on mammary gland morphology was used to assess the apparent ability of CLA to modify the developmental potential of a subset of target cells that are normally susceptible to carcinogen-induced transformation. It was observed that a graded and parallel reduction in terminal end bud density and mammary tumor yield occurred when animals were fed 0.5% and 1% CLA (w/w) in the diet.

Objective 3. ***Does CLA affect the process of clonal selection such that the pathogenetic pathway leading to mammary tumor formation is altered?***

The hypothesis that forms the basis for this objective is that CLA inhibits tumor occurrence by modulating the "activity" of specific genes, whose misregulation is central to the carcinogenic process. The key issue is to identify the genes that CLA modulates, and whether the effect is direct or indirect. During this reporting period methods were developed to study the effects of CLA on the expression of two cell cycle regulatory proteins, cyclin D1 and p27. Data thus far collected suggest that the number of cyclin D1 positive cells is reduced and the number of p27 expressing cells is increased by CLA.

BODY OF PROGRESS REPORT

The effort during the forth year of funding was directed to continuing work on Technical Objective 1, 2 and 3. The following sections detail the methods that were developed to meet the goals stated in these objectives.

Materials and Methods

New Methods Developed During This Reporting Period.

Mammary Gland Epithelial Cell Enrichment We decided to implement a procedure for isolation of mammary epithelial cells from whole gland so as to increase the specificity and sensitivity of our assays of cellular oxidation. We have adopted a modified procedure from Moon, et al (1) in order to efficiently and effectively harvest mammary epithelial cells from the gland.

Approximately a 1 cm square of fresh excised mammary tissue is introduced to 10 mL of sterile incubation medium (medium 199, 2.8% sodium bicarbonate, penicillin streptomycin) and 35 mg of collagenase. This solution is incubated in a Dubnoff shaking incubator at 37^0 for 1-1 1/2 hours with exposure to ultrasonic generator following ½ - ¾ hour of incubation. The mixture is subjected to sonication at 100mA for 2-3 minutes and continues it's incubation in the Dubnoff incubator. The tissue is observed to safeguard against complete disruption which can lead to incomplete separation of epithelial from adipose cells. The mixture is centrifuged at 400 x g for 20 minutes following the transfer into a 15mL centrifuge tube. Proceeding the centrifugation, tissue was observed to be floating in the incubation medium which was removed by aspiration. The walls of the centrifuge tube were cleaned with Kimwipes and the subnatant was exposed to 10 mL of 0.9% sodium chloride solution. The resulting mixture is inverted repeatedly to re-suspend the subnatant and then centrifuged for 10 minutes at 400 x g. Following the fourth wash , the supernatant is carefully aspirated, the walls of the tube wiped with Kimwipes, and the pellet is re-suspended in PBS.

References: Moon, R.C., Janss, D.H., Young, S. (1969) Preparation of Fat Cell-"Free" Rat Mammary Gland. The Journal of Histochemistry and Cytochemistry, 17, 182-186.

Comet Assay For comet analysis, mammary epithelial cells are harvested from the whole gland as discussed above. Cell membranes are further disrupted by use of a non-ionic detergent (nonidet P40) in a dounce homogenizer and nuclei are removed from suspension by centrifugation. Our comet analysis method is based on that of Singh *et al.*(1). Isolated nuclei are suspended in phosphate buffered saline (PBS) at a concentration of ~200,000/ml and then mixed 1:1 with 1.0% low melting point (LMP) agarose in PBS at 37°C. 40 ul of the resulting suspension is quickly delivered onto fully frosted slides precoated with 60ul of 0.5% normal melting point agarose and then covered with a 25mmx30mm #1 coverslip. After the agarose has set a third layer of 40ul of 0.5%LMP agarose is delivered on top and again allow to set. Slides are then incubated in a lysis buffer (1% Triton X-100, 10% DMSO, 1% Na-N-lauroyl sarcosine, 10mM Tris, 100mM Na_2EDTA, 2.5M NaCl, pH 10.0) for one hour. If endonuclease III treatment is desired, slides are washed 3x5min in endonuclease III buffer (.4mM HEPES-KOH, 100mM KCl, .5mM EDTA, .2mg/ml BSA fraction V, pH 8.0) and then covered with 50ul of either endonuclease III in buffer (.05U/ul) or buffer alone, and then incubated for 30 minutes at 37°C. Slides are then placed in electrophoresis buffer (1mM Na_2EDTA, 300 mM NaOH, pH 13.0) for 40 minutes to allow time for DNA unwinding. After unwinding slides are placed in a single row on a horizontal

electrophoresis tank with enough fresh buffer to just cover the slides. After electrophoresis 20min at 25V and 300mA, the slides are neutralized with 3, 5 minute washes (400mM Tris buffer, pH7.5) and then stained with 30ul of a 1/50 dilution of Oli-Green DNA fluorescent dye. Slides are visualized and photographed on a Zeiss Axioskop 20 fluorescence microscope with an excitation filter of 450nm-490nm and a barrier filter of 520nm.

Reference: Sasaki, Y.F., Izumiyama, F., Nishidte, E., Matsusaka, N., Tsuda, S. (1997) Detection of rodent liver carcinogen genotoxicity by the alkaline single-cell gel electrophoresis (COMET) assay in multiple mouse organs (liver, lung, spleen, kidney, and bone marrow). Mutation Research, 391, 201-214.

Assessment of the NF kappa B protein complex and NF kappa B transcriptional activity.

NF kaapa B protein complex by western blot analysis Frozen tissue stored at -80°C is used for protein extraction. 50mg of sample was homogenized in 150ul ice cold RIPA buffer with protease inhibitors (100mM PMSF, 1.9ug/ml Aprotinin, 1M DTT). The volume was brought to 1ml with ice-cold RIPA. The homogenate was incubated on ice for 30 minutes. Sample was centrifuged 2X for 20 minutes and 1X for 5 minutes to obtain a clear supernatant of whole cell lysate (16,000Xg, 4°C). Supernatant was frozen and stored at -80°C. Protein was quantified using Bio-Rad Bradford assay kit. We used a dose response, run on a 10% protein gel at 0, 5, 10, 20, and 40ug, to decide that 40ug of protein was the optimum to conserve antibody and to concentrate any less abundant proteins. The dose response with duplicate loading was also used to determine our reproducibility. Samples were run on a 10% running gel (37.5:1 bis/acrylamide) and a 5% stacking gel. Protein samples were added to 15ul 2X loading buffer and denatured by boiling 5 minutes and placed on ice. Rainbow marker (Amersham RPN 756) and mammary tumor protein was used as positive controls. After exposure, each membrane is stripped and re-hybridized for -actin to confirm gel loading and sample integrity. Proteins were transferred from gel to nitrocellulose membrane by semi-dry transfer method. All incubations are performed in a hybridization oven at 37°C. 5% milk solution is prepared with non-fat dry milk in 1XTBS/0.1% Tween-20 (TTBS) solution and used for blocking (1hour) and antibody dilutions. Then, membrane was blocked with 20ml of milk solution for 1 hour. Add primary antibody in 5ml 5% milk solution and incubate for 1 hour. All antibodies were ordered from Santa Cruz and used at the following dilution factors: NF B p65 at 1:1000, IkB- at 1:200, -actin at 1:1000. We are trying to achieve better results for NF B p50. Wash membrane 3X with 15ml of TTBS. Add HRP-conjugated secondary antibody for 1 hour (Sigma anti-rabbit IgG 1:10K). Wash membrane 3X with 15ml of TTBS. ECL was performed using Amersham kit (RPN 2106).

Detection of DNA-protein interaction by electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA)
Proteins from cell extracts from either the nucleus or cytoplasm are detected by their ability to retard mobility of a labeled DNA fragment during electrophoresis through a nondenaturing gel. This method is known as **electrophoretic mobility shift assay (EMSA)**. The protocol of EMSA include four stages:

- Preparation of the nuclear or cytoplasmic extracts from mammalian cells
- Preparation of a radioactivity labeled DNA probe containing a particular protein binding site
- A binding reaction in which a protein mixture is bound to the DNA probe
- Electrophoresis of protein-DNA complexes through the nondenaturing gel, which is then dried and autoradiographed

We are using Promega's Gel Shift Assay System (Promega Catalog # E3300). Nuclear extracts are prepared from fresh rat tissue using the protocol in Current Protocol in Molecular Biology (edited by Ausubel, F.M., et al) with minor modifications. NF-κB consensus oligo (Promega Catalog # E3291) is labeled by T_4 polynucleotide kinase reaction with γ-^{32}PdATP (3000 Ci/mmol, 10 μCi/μl). Unincorporated label is removed by ethanol precipitation. In our DNA binding reactions, competition assays, i.e., negative control (probe along), positive control (using Hela nuclear extract, Promega Catalog # E3521), specific competitor (unlabeled competitor oligo) and nonspecific competitor (unlabeled non competitor) are included. Competition mobility shift assay and antibody super shift assays are used because most protein preparations will contain both specific and nonspecific DNA binding proteins. The competition shift assay will also use a NF-κB mutant oligonucleotide (Santa Caruz Biotechnology Catalog # sc-2511) which is identical to the probe fragment except for a mutation in the binding site that is known to disrupt function and presumably binding activity. In brief, 2 μl of Hela nuclear extract or 12-15 μg crude extract from rat tissue is incubated with 5,000-20,000 cpm radiolabeled NF-κB probe, and appropriate competitor in 1X Gel Shift Binding Buffer in a volume of 10 μl. The protein should be added last. The reaction is incubated at 30 ° for 30 min. 1 μl of 10X loading buffer is added to the negative control and 1 drop of glycerol is added to rest of the reactions. The protein-DNA complexes are electrophoresized on a prerunned 4% nondenaturing polyacrylamide gel (acrylamide:bisacrylamide=37.5:1) with 1.5 mm thick spacers and comb in 1XTBE buffer until the appropriate separation of bands is achieved. The gel is run at 200 V in a cold room to minimize overheating and denaturation of the probe during electrophoresis. The gel is then transferred to a filter paper, dried on a gel dryer, and exposed to X-ray film at -80°C with an intensifying screen.

Antibody supershift assay If the protein that forms the complex is recognzed by the antibody, the antibody can either block complex formation, or it can form an antibody-protein-DNA ternary complex and thereby specifically result in a further reduction in the mobility of the protein-DNA complex. In the present work, antibodies of NF-κB p65 (A), p65 (C-20), p50 (NLS), p50 (D-17) and p52 (K-27) from Santa Cruz (Catalogue # sc-109-G, sc-372-G, sc-114-G, sc-1192, and sc-298) are used for the supershift assay.

Measurement of mammary gland growth All whole mounts of the abdominal-inguinal mammary gland chains were photographed at 2 X magnification and the resulting images were scanned and digitized. Measurements of ductal extension of the mammary gland into the fat pad and of the amount of ductal branching were performed on the digitized images of entire abdominal-inguinal mammary gland chains using "IMAGE-PRO PLUS" software (Media Cybernetics). The length of mammary gland between the upper most lymph node and mammary branch border is quantified as a measure of ductal extension (DE). Images are then further processed to remove the lymph nodes and lesions from the mammary gland. This processed image is then evaluated for total area of the mammary gland fat pad into which mammary ductal epithelium has extended. This area is defined by drawing a line around the 360 degree perimeter of a mammary gland chain established by the point to point connection of a line from the outer most extending end bud one to the other. The area within this perimeter is then determined by subtracting the area occupied by the lymph nodes and lesions. We next use digital filters to assess the percent of this area that is occupied by mammary epithelium, i.e. the amount of ductal branching.

BrdU labeling and counting A pulse labeling technique is used to assess the rate of cell proliferation. Rats are injected with 50 mg bromodeoxyuridine/kg body weight i.p. exactly 3 hrs prior to euthanasia. The three hour period of labeling is selected since evidence indicates that this also is the time span represented in the quantification of cell death by apoptosis using

morphological criteria. Uninvolved mammary gland duct and pre-malignant and malignant mammary gland lesions are stained for immunohistochemical analysis. Anti-BrdU antibody (Becton Dickinson, 1:40) was used to detect BrdU labeled nuclei. Labeled and unlabeled cells are imaged and counted at 400X with a computer assisted image analyzer (CAS-200), using the Quantitative Nuclear Antigen Program Version 3.0 (Becton-Dickinson/Cellular Imaging Systems, San Jose, CA). Two cameras with two bandpass filters, one at 620 nm, which measures all nuclei stained with hematoxylin (with or without DAB staining) and the other at 500 nm, which measures only nuclei stained with DAB, allow for excellent spectral discrimination between the brown (DAB chromoagen) and blue (hematoxylin). Both nuclear and antibody thresholds are set with a negative control antibody cocktail slide. The nuclear threshold is set to the value that best discriminates between the nuclei and cytoplasm. The antibody threshold is set to the value at which no stain can be detected in the nuclei of the negative control slide. Standardization is established through the use of control tumor tissue in each assay, which is treated in the same manner as the sample tissue. The Quantitative Nuclear Antigen software application is used to measure the percentage of cell nuclei in the tissue sections that contain BrdU labeled antigen. The proliferation index, the percentage of labeled cells over total cells counted, is determined by counting 20 fields per each slide (approximately 2000 cells).

Apoptotic cell counting The same sections used for determining proliferation index are stained using a standard H&E protocol. Apoptotic cells are identified by morphological criteria. The apoptotic index, the percentage of apoptotic cells over total cells counted, was determined on the same 20 fields subjected to analysis for cell proliferation.

Cyclin D1 staining and quantification Uninvolved mammary gland duct and pre-malignant and malignant mammary gland lesions are stained for immunohistochemical analysis of cyclin D1. Anti-cyclin D1 antibody (NeoMarkers, 1:40) is used to detect cyclin D1 labeled nuclei. Labeled and unlabeled cells are imaged and counted at 400X with a computer assisted image analyzer (CAS-200), using the Quantitative Nuclear Antigen Program Version 3.0 (Becton-Dickinson/Cellular Imaging Systems, San Jose, CA) (xx). Two cameras with two bandpass filters, one at 620 nm, which measures all nuclei stained with hematoxylin (with or without DAB staining) and the other at 500 nm, which measures only nuclei stained with DAB, allow for excellent spectral discrimination between the brown (DAB chromoagen) and blue (hematoxylin). Both nuclear and antibody thresholds were set with a negative control antibody cocktail slide. The nuclear threshold was set to the value that best discriminated between the nuclei and cytoplasm. The antibody threshold was set to the value at which no stain could be detected in the nuclei of the negative control slide. Standardization is established through the use of control tumor tissue in each assay, which is treated in the same manner as the sample tissue.

The Quantitative Nuclear Antigen software application is used to measure the percentage of cell nuclei in the tissue sections that contain cyclin D1 labeled antigen by counting 20 fields per each slide (approximately 2000 cells), and three parameters were taken. These are: (1) positive area, which means the percentage of the nuclear area that is positive for the cyclin D1; (2) positive stain, which represents the summed optical density for the positive area divided by the summed optical density for the entire nuclear area, and give an indication of the cyclin D1 density in the positive area; (3) positive optical density (POD) per positive cell (PC), which represents the average summed optical density for the cyclin D1 staining within each cell, and indicates the amount of cyclin D1 per each cell.

P27 staining and quantification Uninvolved mammary gland duct and pre-malignant and malignant mammary gland lesions are stained for immunohistochemical analysis of p27. Anti-p27 antibody (NeoMarkers, 1:40) is used to detect p27 labeled nuclei. Labeled and unlabeled cells are imaged and counted at 400X with a computer assisted image analyzer (CAS-200),

using the Quantitative Nuclear Antigen Program Version 3.0 (Becton-Dickinson/Cellular Imaging Systems, San Jose, CA) (xx). Two cameras with two bandpass filters, one at 620 nm, which measures all nuclei stained with hematoxylin (with or without DAB staining) and the other at 500 nm, which measures only nuclei stained with DAB, allow for excellent spectral discrimination between the brown (DAB chromoagen) and blue (hematoxylin). Both nuclear and antibody thresholds are set with a negative control antibody cocktail slide. The nuclear threshold was set to the value that best discriminated between the nuclei and cytoplasm. The antibody threshold was set to the value at which no stain could be detected in the nuclei of the negative control slide. Standardization was established through the use of control tumor tissue in each assay, which is treated in the same manner as the sample tissue.

The Quantitative Nuclear Antigen software application is used to measure the percentage of cell nuclei in the tissue sections that contain p27 labeled antigen by counting 20 fields per each slide (approximately 2000 cells), and three parameters were taken. These are: (1) positive area, which means the percentage of the nuclear area that is positive for the p27; (2) positive stain, which represents the summed optical density for the positive area divided by the summed optical density for the entire nuclear area, and give an indication of the p27 density in the positive area; (3) positive optical density (POD) per positive cell (PC), which represents the average summed optical density for the p27 staining within each cell, and indicates the amount of p27 per each cell.

Source and composition of CLA and other dietary fats. The method of CLA synthesis from 99+% pure linoleic acid is detailed in reference (6, listed above). CLA was custom ordered from Nu-Chek, Inc. (Elysian, MN). Gas chromatographic analysis showed that three particular isomers, *c*9,*t*11-,*t*9,*c*11- and *t*10,*c*12-CLA, constituted about 90% of the total. There were minimal variations in isomer distribution from batch to batch. Other fats used included: Mazola brand corn oil was obtained from Best Foods, Somerset, NJ, lard was purchased from Harlan Teklad, Madison, WI, menhaden oil was obtained from Marine Oil Test Program, U.S. Department of Interior, and palm oil was obtained from the Edible Oils Institute.

Animals and Diets

Animals. Female Sprague Dawley rats were used in the work reported. They were obtained from either Taconic Farms (Germantown, NY) or Charles River, Wilmington, Delaware. All rats were certified pathogen free.

Diet Formulations. A variety of diet formulations were used depending on the research question being addressed. All diets were modifications of the AIN-76A formulation and were designed to meet or exceed the known nutrient requirements of the rat unless otherwise specified.

Previously Reported Methods The Use of Which Continues.

Analysis of urinary malondialdehyde (MDA). Following acid hydrolysis to release the bound form, MDA was derivatized with thiobarbituric acid (TBA) and the MDA-TBA adduct quantified by reverse phase HPLC with visible absorbance detection at 535nm. MDA content is expressed as nmol/mg creatinine.

In detail, 0.5 ml urine was combined with 5ul of an antioxidant solution containing 0.3M 2dp and 2% BHA in ethanol, and 40 ul concentrated HCL. The mixture was heated in a dry block at 96-99° for 4 and 3/4 hours. After samples had cooled slightly, 2 ml of TBA solution (1.11 % TBA in 74mM KOH) was added and the samples were heated at 96-99° for another 45

minutes. After cooling and immediately before HPLC analysis, samples were adjusted to a pH of 1.8 - 4.0 with 12N KOH. Previous method validation has confirmed that the presence of 2dp and BHA in urine samples during acid hydrolysis and TBA derivitzation prevents artifactual MDA contribution from food contamination in the urine, even with extreme contamination by menhaden oil containing diet. Creatinine was measured spectrophotometrically (Procedure 555, Sigma Diagnostics, St. Louis, MO 63178).

Determination of 8-OHdG and malondialdehyde in mammary tissue.

8-OHdG. For the assay of 8-OHdG, the various procedures of DNA purification from the mammary gland, the enzymatic digestion of DNA to deoxynucleosides, the isocratic separation of 8-OHdG and dG by HPLC, and the quantitation of 8-OHdG with an electrochemical detector were described in detail in a recent publication from our laboratory (11). Detector response was linear from 10 to >800 pg per injection for 8-OHdG and from <500 to 6000 ng for dG. Results are reported as residues of 8-OHdG per 10^6 residues of dG. The simultaneous analysis of both deoxynucleosides on a single HPLC injection abrogated the need for a recovery standard.

Malondialdehyde (MDA). Tissue malondialdehyde was quantified as its thiobarbituric acid derivative with reverse phase HPLC and photometric absorbance detection at 535nm. In detail, mammary gland was homogenized with a Polytron in water containing 1% antioxidant solution (AOS: 0.3M dipyridyl and 2% BHA, in ethanol), 1 part mammary gland to 9 parts water (wt/vol). Homogenized samples were centrifuged at 6500 x g and fat plugs were removed, followed by further homogenization to re-suspend the pellet. As optimum reaction conditions were found to vary with protein concentration, an amount of homogenate containing approximately 1.25 mg protein was prepared for hydrolysis. The homogenate was combined, in glass tubes, with 7.5 ul AOS and enough water to bring the volume to 1.47 ml. 7.5 ul 5N HCl was added, and covered tubes were heated to 96° C for 3 hours. Tubes were cooled quickly in tap water, and 30 ul sodium tungstate (Na_2WO_4) per tube was added to facilitate precipitation of protein. Tubes were centrifuged at 6500 x g for 10 min, and 1 ml of supernatant was then transferred from each to clean glass tubes. (The remaining supernatant and pellet were discarded.) 0.75 ml thiobarbituric acid (TBA) solution (1.11% TBA in 74 mM KOH) was added to each tube, and tubes were heated for 90 min for derivatization (to form TBA-MDA adduct). Samples were quickly cooled and the pH adjusted, if necessary, to between 2.5 and 4.0. The MDA-TBA adduct was separated using a 4.6 x 150 mm C18 column (Beckman Ultrasphere ODS) and a mobile phase consisting of 32.5% methanol in 50mM potassium phosphate buffer, pH 6.0 delivered at 1.5 ml/min. Photometric absorbance detection was at 535nm. MDA was quantified by comparison of sample peak heights to those of standards, prepared from 1,1,3,3-tetramethoxypropane (TMP). To aliquots of stock standard were added water to 1.5 ml, 5 ul AOS, 1 ml TBA solution and 40 ul concentrated HCl. Standards were heated at 96° C for 14 min, cooled, and their pH adjusted to between 2.5 and 4.0 with 12N KOH. Final results were expressed as nmol MDA/mg protein. Protein in tissue homogenates was quantified by the Bradford method using a commercial dye reagent (Bio-Rad Protein Assay, Bio-Rad Laboratories, Richmond, CA).

Determination of 8-OHdG concentration in liver DNA . The procedures described exhaustively herein contain significant changes from those previously described by us. The changes, such as eliminating phenol from the DNA isolation and adding BHT and 2-dp to buffers have been instrumental in reducing the contribution of artifacts to measured 8-OHdG. The importance of guarding against artifacts and their mistaken interpretation can not be over stated.

Isolation and enzymatic digestion of DNA from rat liver. DNA was isolated from liver with a

phenol free process and was subsequently digested enzymatically to nucleosides for chromatographic analysis. In detail, 10ul of 26.4 mg/ml BHT was added to a 13 ml polypropylene screw cap tube, followed by 3 ml digestion buffer (100mM NaCl; 10mM Tris, pH8.0; 0.5% sodium dodecyl sulphate, pH 8.0, 400 ug/ml proteinase K (30 mAnson units/mg, cat # 24568, EC 3.4.21.14, from EM Science)) and approximately 75 mg frozen pulverized liver. The tube was inverted repeatedly to mix and incubated in a 50° water bath for16-20 hrs, after which it was removed from the bath and allowed to cool briefly before adding 1 ml 7.5M ammonium acetate and mixing thoroughly. The resulting precipitate was removed from suspension by centrifugation at 19000g for ten minutes at 4°, and the supernatant decanted and extracted twice with 24:1 chlroform/isoamyl alchohol. Nucleic acids were precipitated by the addition of 3 ml isopropanol, transferred to 1 ml silanized glass vials (Type I, Class A borosilicate glass, Waters Associates, Milford, MA) and the precipitate was washed with 70% EtOH before dissolution in 340 ulTE buffer (10mM Tris;`1mM EDTA; pH 8.0) containing 5mM dp. RNA contamination was reduced by treating samples with RNase (55 ug in H_2O) for 1 hour at room temperature in the dark. After addition of 10 ul of 5M NaCl, DNA was precipitated by the addition of 350 ul isopropanol. While the presence of ribonucleosides does not interfere with the assay per se, removal of most of the RNA by treatment with RNase results in samples which are more readily digested to nucleosides and chromatographed. The DNA pellet was washed with 70% EtOH, dried briefly under reduced pressure without heat, and dissolved in100ul of 20mM sodium acetate, pH 4.8, containing 5mM DP. Dissolution was allowed to proceed overnight at room temperature in the dark prior to enzymatic digestion to nucleosides.

Chromatography of liver hydrolysate. 8-OHdG and dG were separated isocratically on a 4.6 X 250 mm Rainin Microsorb C18 column (5um, 100A) with a mobile phase of 8.2% methanol in 50 mM potassium phosphate buffer, pH 5.5, delivered at 1 ml/min. Detection of 8-OHdG was achieved on an ESA Coulochem Model 5100 A electrochemical detector equipped with a model 5011 analytical cell and a model 5020 guard cell. Detector potentials were set as follows: guard cell +0.43 V, detector one +0.12 V, detector two +0.38 V. 8-OHdG was measured as current at detector two. dG was monitored by absorbance at 290 nm with a Shimadzu SPD-10AV spectrophotometric detector installed downstream from the electrochemical detector. Results were reported a residues 8-OHdG per million residues dG. The simultaneous analysis of both analytes from a single HPLC injection provided excellent precision without rigorously quantitative sample handling.

RNA isolation Total RNA was extracted from carcinomas and tissues by acidic phenol extraction using a commercial kit from BIOTECX Laboraotries, Inc. (Houston, TX). For differential display of mRNA, the total RNA preparations were digested with RNase-free DNase (GenHunter Corporation, Nashville, TN) in order to remove contaminating genomic DNA. For cDNA library construction, poly A(+) mRNA was enriched by oligo (dT)-cellulose column.

Differential display Differential display of mRNA was carried out with the RNAimage™ kit (GenHunter Corporation, Nashville TN) according to manufacturer's instructions with two minor modifications: 1) One tenth of the recommended amount of total RNA was used for the reverse transcription step in order to minimize an inhibitory activity(s) present in the mammary tissue RNA preparation; 2) It was found that an annealing temperature of 42 °C for PCR was optimal in Denver, CO to yield reproducible display patterns. Duplicate reactions were run for each primer combination. The PCR products (labeled by alpha-^{32}P-dATP) from the 3 mammary carcinomas (T_1, T_2 and T_3), the uninvolved mammary tissue and kidney were contrasted side-by-side on sequencing gels. Only those bands that were present in carcinoma lanes, but absent in mammary gland and kidney lanes were cut and re-amplified by PCR. The PCR products were size separated on low melting point agarose gel and band(s) of the expected size was eluted. The gel-purified PCR bands were used as templates to generate ^{32}P-labeled

probes for Northern blot detection of gene expression on a screening panel of RNA preparations comprised of two kidney samples, two liver samples, the 3 mammary tumors that were used for the original differential display. In addition, the uninvolved mammary gland tissue and mammary gland tissue excised from a day-1 post-partum female rat were included in the screening panel. The RNA samples were size-separated by electrophoresis and transferred onto Nylon membrane for Northern blot detection of gene expression. GAPDH or cyclophylin gene was probed as an internal control for loading correction.

Cloning and Sequencing Those PCR bands that detected carcinoma-specific gene expression were cloned into pGEM-T vector (Promega, Madison, WI). For each band, four clones were inoculated and the plasmid DNA was isolated by an alkaline mini-prep procedure and at least two clones are sequenced on both strands by the dideoxy chain termination method of Sanger (7) using a kit from US Biochemicals (St Louis, MO). A commercial service utilizing thermal cycle sequencing (Cornell DNA Service, Ithaca, NY) was also used to confirm the sequence of a few of the clones. The cloned gene fragments were used as templates to generate randomly labeled probes for Northern detection again to confirm that the cloned sequences corresponded to the genes originally detected by the PCR products from differential display gels. Sequence search was done using the BLASTN algorithm (8) with GenBank nr databases and expressed sequence databases dbEST.

Cloning full length cDNA A cDNA library was constructed with pooled poly(A)+ mRNA isolated from mammary carcinomas using the Marathon cDNA construction kit (Clontech, Inc, Palo Alto, CA). The average length of the library was 1.5 kb. Based on the sequence information obtained for each gene fragment, a gene-specific primer was synthesized (Integrated DNA Technology, Inc, Coralville, IA) as the down stream primer. A universal upstream primer that annealed to the adapter which had been ligated into the cDNA library and the gene-specific primer were used for long distance PCR using KlenTaq (a combination of Taq and Vent polymerases) to increase fidelity of cloning (ClonTech, Palo Alto, CA). The PCR fragments were cloned into the pGEM-T vector and sequenced as described above.

Ha-*ras* codon 12 mutation detection in mammary carcinomas The paraffin-embedded tumor blocks were cut into 5-*um* sections. These sections were mounted on plastic slides coated with polylysine and stained with hematoxylin and eosin (H&E). Each section was viewed without a cover slip under light microscopy and marked for tissue retrieval. Pieces of a section (approximately 2x2 mm) were carefully cut with scissors from the marked area. The scissors were soaked in 10% Chlorox bleach and heat sterilized between samples to prevent carry-over. Each piece was incubated with proteinase K (400 :g/ml in 100 mM Tris-HCl, 2 mM EDTA) at 50°C for 3 h. After the proteinase K was inactivated by heating at 95°C for 8 min, 5%-10% of the extract was used as the source of DNA for PCR amplification. This tissue collection procedure permits sampling of different regions of a carcinoma for PCR analyses. It is important to note that when this tissue sampling procedure was tested in independent experiments more than one hundred samples have been repeated at different times to check the reproducibility of the assay and to ensure the absence of carry-over and all of the repeated measures showed reproducible results.

The mutational status of Ha-*ras* codon 12 was determined by a modified polymerase chain reaction-generated restriction fragment length polymorphism (PCR-RFLP) method (16,17). The upstream primer (5'AGTGTGATTCTCATTGGCAG-3') was placed in intron-1 to avoid amplifying the Ha-*ras* pseudogene. The G → A mutation along with two mismatches in the downstream primer (5'-AGGGCACTCTTTCgaACGCC-3') introduces a XmnI restriction site into the PCR product (116 bp), which, upon digestion with the XmnI endonuclease of the PCR products, generates a fragment of 98 bp that is diagnostic for the mutation. A tracer amount of alpha-^{32}P-dCTP was used to label the PCR products. The digested products were separated by

electrophoresis on a 6% polyacrylamide gel and detected by autoradiography using Kodak X-ray films.

Results and Discussion

Further analyses of the cellular effects of CLA on oxidative damage indices

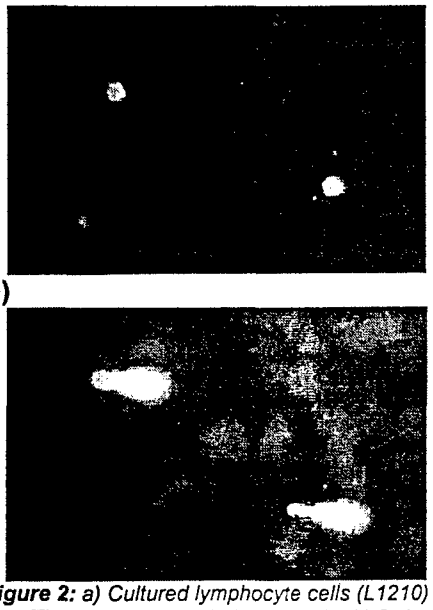
We have reported that feeding CLA reduced lipid peroxidation in mammary gland measured as malondialdehyde but had not effect on DNA oxidation measured as 8-OHdG.This was an unanticipated observation. Given the heterogeneity of the mammary gland and the potential loss of sensitivity to detect small but meaningful changes in oxidative DNA base damage in the target mammary epithelial cells, we undertook the development of methods necessary to specifically evaluate the question, "Does CLA protect mammary epithelial cells against oxidative DNA base damage? After considering several approaches we decided to isolate epithelial cells from mammary gland and then subject them to a single cell electrophoresis analysis for assess oxidative DNA base damage. In following paragraphs we report our progress in assay development.

We have developed a modified alkaline single cell gel electrophoresis assay (comet assay) to estimate DNA damage in nuclei isolated from mammary gland tissue. Our method is a variation of that described previously by Singh *et al.* (1). Briefly, nuclei are lysed with high ionic strength buffer containing ionic and non-ionic detergents after embedding in a thin agarose gel on microscope slides. Alkaline conditions facilitate unwinding and areas of relaxed supercoiling in DNA that has been damaged by strand breaks. During electrophoresis, the free ends and relaxed loops of DNA are free to migrate towards the anode, producing a tail (thus the name "comet assay") when visualized after staining with a fluorescent dye ***(figure 1)***. We have chosen to use the fluorescent DNA dye Oli-Green (Molecular Probes) due to its high affinity for ssDNA. In addition to detecting frank breaks and alkali labile sites we have used a DNA repair enzyme to detect specific oxidative damage. Incubation with endonuclease III converts specific oxidative damage to strand breaks by excising oxidized pyrimidines (3).

Figure 1: *Enlarged image of a comet from cultured lymphocytes (L1210 cell line). This image shows the strongly fluorescent nucleus/head and the tail produced by free ends and relaxed loops of DNA after electrophoresis.*

As a "positive control" in assay development we have shown that treatment of cultured L1210 cells with H_2O_2 results in comets that are readily distinguishable from untreated controls by visual scoring ***(figure 2)*** and we have observed a positive dose/response correlation between H_2O_2 treatment and comet tail magnitude as well. However, a more precise estimation of DNA damage via comet assessment is anticipated for achieving the goals of this proposal. To that end, we are currently evaluating specialty software that facilitates a more discriminating estimation of DNA damage based on comet tail moment. Tail moment is a useful index of DNA damage that offers increased sensitivity and precision over other methods such as categorical scoring and tail length. Digital evaluation of tail moment has been shown to achieve even greater sensitivity(2).

a)



b)

Figure 2: *a) Cultured lymphocyte cells (L1210) compared to b)cultures lymphocyte cells treated with 200uM H_2O_2 for 5 minutes on ice. The DNA damage induced by the H_2O_2 is clearly seen by the extensive tails in figure 2b).*

Because of the relative homogenity of liver we next applied the comet analysis to nuclei isolated from frozen solid tissue (rat liver) has shown extensive tailing that is inconsistent with expectation. We suspect this extensive tailing is due to artifacts of tissue preparation, probably from freezing. Data acquired from comet analysis of cultured lymphocytes that have been frozen in 10% DMSO indicate that frozen cells can yield comets with tails of low intensity, but that cells must be frozen in media or buffer containing DMSO such that membrane disruption and DNA strand breakage are minimized. Upon thawing, properly frozen samples show ~28% dead cells by trypan blue dye exclusion criteria, and subsequent analysis reveals two distinct populations of comets, one of which shows minimal comet tail magnitude and one consisting almost entirely of tails ***(figure 3)***. The relative proportions of these populations is comparable to those of live and dead cells respectively. In contrast, cells from fresh blood uniformly have minimal comet tails prior to endonuclease III treatment. Thus it appears that while cells from fresh tissue are optimal for comet analysis, the ability to identify and exclude dead cells from analysis will facilitate frozen storage of samples if such storage is necessary.

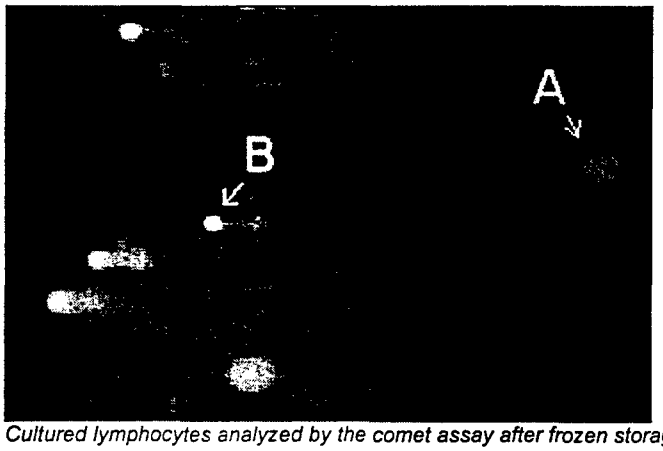


Figure 3: *Cultured lymphocytes analyzed by the comet assay after frozen storage in 10% DMSO. Examples of the two cell populations are labeled:* A *indicates a comet which is primarily tail and* B *indicates a comet with minimal damage.*

Current Status During the summer of 1998 our laboratory began interacting with the laboratory of Dr. Andrew Collins in Scotland. That laboratory has extensive experience in the use of the comet assay. Because of "quirks" of the assay as applied to tissue, we decided to send one of our technical staff to Scotland to work in Dr. Collins' lab. This activity will occur at the end of October 1998. Following that visit we will continue our work on this aspect of the project. Presuming that assay variability can be adequately controlled, we will complete work on this aspect of the project during the one year no cost extension of this project.

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Possible role of NF-κB in reduction of breast cancer risk by increased consumption of CLA

Our working hypothesis is that CLA increases the "reduced state" of the cell and thereby reduces the risk of mammary carcinogenesis by decreasing the transcriptional activation of NF kappa B. We anticipate that the comet assay described above will provide evidence of an "oxidation protected state" in mammary epithelial cells.

What is NF-κB? Originally defined as the nuclear factor that bound to the B site of the immunoglobin κ light chain gene enhancer in B lymphocytes, NF-κB is now known to be a family of dimeric transcription factors, with subunits that contain an amino-terminal stretch of approximately 300 amino acids that share homology with the v-Rel oncogene. Classical NF-κB is composed of a p50 and a p65 subunits. In addition, other Rel-related subunits have been identified, including c-Rel, RelB, and p52.

NF-κB is normally found in the cytosol as an inactive complex consisting of two subunits, p50 and p65, which are bound to an inhibitory subunit, termed IκB. Other members of the rel transcription factor family can also contribute to NF-κB complexes. Upon activation, NF-κB is released from IκB and translocates to the nucleus, where it binds its cognate DNA sequences and increases the transcription of specific genes.

Reactive oxygen intermediates and NF-κB Reactive oxygen intermediates (ROI), including hydrogen peroxide (H_2O_2) have been demonstrated to be potent activators of NF-κB. Consistent with these findings, antioxidants are effective inhibitors of NF-κB. In addition, administration of H_2O_2 and overexpression of glutathione peroxidase can each lead to induction or inhibition, respectively, of NF-κB activity. Therefore, the NF-κB activation pathway appears to be upstream of oxidative stress and ROI.

In a mouse model of aging, NF-κB was found to exist in a constitutively activated state in cells obtained from the major lymphoid organs of aged animals. Therapeutic treatment with dietary antioxidants or with agents capable of activating the peroxisome proliferator-activated receptor (PPAR)-α was able to correct the abnormal nuclear NF-κB activity, reduce lipid peroxide levels, and eliminate the dysregulated expression of cytokines and other genes under NF-κB control. These results suggest that abnormal activation of NF-κB in aging contributes to the dysregulated expression of certain pleiotropic cytokines.

How does modulation of NF-κB activity contribute to cancer risk?

Regulation of c-myc oncogene promoter by the NF-κB rel family

The promoter of the c-myc protooncogene contains two NF-κB binding sites. The effects on activation of a c-myc promoter/exon 1-CAT construct in NIH 3T3 cells by the individual members of the NF-κB family were tested. Classical NF-κB (p65/p50) was found to be a potent transcriptional activator of the c-myc promoter. Cotransfection with either p65 alone or p65 in combination with p50 mediated significant induction. In contrast, expression of either v-rel or chicken c-rel failed to transactivate, while murine c-rel induced c-myc promoter activity only slightly. Furthermore, induction by classical NF-κB was inhibited by coexpression of either v-rel or chicken c-rel. Thus, individual members of the rel family have differential effects on the c-myc oncogene promoter, which can modulate overall transcription activity and allow for precise regulation of this oncogene under diverse physiologic conditions. Activation of NF-κB may prevent c-myc-induced apoptosis, allowing neoplastic progression. NF-κB is activated in many different cell types by a variety of agents, suggesting that this factor is an important global transcriptional regulator, which is induced by numerous stimuli.

Several reports have suggested that proteins from the NF-κB or IκB families are involved in the development of cancer. v-Rel expressing viruses are highly oncogenic and cause aggressive lymphomas in young birds, while mutated c-Rel is transforming *in vitro*. The genes coding for c-Rel, p65, p50, p52 and Bcl-3 are located at sites of recurrent chromosomal translocations and genomic rearrangements in human cancers.

Increasing evidence supports the role of NF-κB/Rel family involvement in apoptosis. NF-κB controls the expression of growth factors or oncoproteins (c-Myc, Gro proteins), as well as of proteins derived from tumor suppressor genes such as p53. Therefore it is possible that, in different cellular environments, upregulated activation or inhibition of NF-κB activity could each lead to tumor formation. NF-κB also regulates the transcription of genes coding for extracellular proteases or adhesion molecules (urokinase, 92 kD type IV collagenase), and could therefore play a role in angiogenesis or invasiveness, two mechanisms required for tumor growth and the development of metastasis.

Detection of DNA-protein interaction by electrophoretic mobility shift assay

Proteins from cell extracts from either the nucleus or cytoplasm are detected by their ability to retard mobility of a labeled DNA fragment during electrophoresis through a nondenaturing gel. This method is known as **electrophoretic mobility shift assay (EMSA)**. The protocol of EMSA include four stages:

- Preparation of the nuclear or cytoplasmic extracts from mammalian cells
- Preparation of a radioactivity labeled DNA probe containing a particular protein binding site
- A binding reaction in which a protein mixture is bound to the DNA probe
- Electrophoresis of protein-DNA complexes through the nondenaturing gel, which is then dried and autoradiographed

The sensitivity of the EMSA enables femtomole quantities of DNA-bind proteins to be detected. This assay can be used to test binding of purified proteins or of uncharacterized factors found in the crude extracts. It also permits quantitative determination of the affinity, abundance, association rate constants, dissociation rate constants, and binding specificity of DNA-binding proteins.

Three additional protocols are available for competition assays to assess the sequence specificity of protein-DNA interactions. This is necessary because most protein preparations will contain both specific and nonspecific DNA binding proteins.

- Competition mobility shift assay
- Antibody supershift assay
- Multicomponent gel shift assays

Questions under investigation:

- Does increased intake of CLA reduce DNA damage measured by COMET assay?
- Does increased intake of CLA exert a cancer-protective effect via modification of DNA-binding activity of NF-κB?
- Are levels of oxidative DNA base damage as measured by COMET assay positively associated with DNA-binding activity of NF-κB?

Current Status As indicated above we intend to complete work on this element of the grant during the one year no cost extension of the project.

Additional findings Since the submission of the last progress report we have submitted for review a paper entitled, "The signficance of a decrease in linoleic acid metabolites as a potential signaling mechanism in cancer risk reduction by conjugated linoleic acid". This was a collaborative effort with the laboratories of Sebastiano Bani and Clement Ip. Briefly, we report in that paper that CLA induced a graded and parallel reduction in mammary gland density and mammary tumor yield at 0.5 and 1% in the diet (w/w). No further decrease in either parameter was observed when CLA in the diet was raised to 1.5 or 2%. Since CLA and linoleic acid are likely to share the same enzyme system for chain desaturation and elongation, it is possible that increased CLA intake may interfere with the further metabolism of linoleic acid. Fatty acid analysis of total lipid showed that CLA and CLA metabolites continued to accumulate in mammary tissue in a dose-dependent manner over the range of 0.5% to 2% CLA. There was no purturbation in tissue linoleic acid; however, linoleic acid metabolites (including 18:3, 20:3 and 20:4) were consistently depressed by up to 1% CLA. There seemed to be a plateauing effect above this level. Of particular interest was the significant drop in 20:4 (arachidonic acid) which is the substrate for the cyclooxygenase and lipoxygenase pathways of eicosanoid biosynthesis. Thus the CLA dose response of arachidonic acid suppression corresponded closely with the CLA dose response of cancer protection in the mammary gland. These data provide new insights about the biochemical action of CLA.

CONCLUSIONS

Conjugated linoleic acid (CLA) is a naturally occurring component of the food supply that has been shown to inhibit the development of experimentally-induced breast cancer. During this reporting period we have obtained data that indicate that exposure to CLA feeding during the time of mammary gland maturation may modify the developmental potential of a subset of target cells that are normally susceptible to carcinogen-induced transformation. We are

currently investigation the role of CLA in modifying the transcriptional activation of redox sensitive proteins and in the regulation of cell cycle transit.

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